Increased Numbers of Dendritic Cells in the Bronchiolar Tissues of Diffuse Panbronchiolitis

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Dendritic cells (DCs) are potent antigen-presenting cells (APCs); they are considered to be the most important APC in the lung. Recently, the number of DCs in the large airways was demonstrated to increase in patients with atopic asthma, leading to the concept that DCs play an important role in airway inflammation. However, little is known about the distribution of lung DCs in the small airways under other pathological conditions. The aim of the present study was to examine the distribution of DCs in the bronchiolar tissues in patients with diffuse panbronchiolitis (DPB), which is a chronic inflammatory disorder of the airways histologically characterized by peribronchiolitis. We investigated the distribution of DCs in the bronchiolar tissues of the lungs in 11 patients with DPB and 7 control subjects with normal lungs using immunohistochemical methods. Marked increases in the number of $CD1a^+$, $CD1c^+$, and $CD83^+$ DCs were found in both the bronchiolar epithelium and submucosal tissues of patients with DPB, compared with control subjects with normal lungs. The most striking increase occurred in the number of DCs expressing CD83, a marker of mature DCs, in the submucosal tissues of patients with DPB. The increases of these positive cells in patients with DPB were more marked in the submucosal tissues than in the epithelium. The bronchiolar epithelial cells in patients with DPB strongly expressed GM-CSF protein, which is an important cytokine for the differentiation and function of DCs, suggesting that the increased local production of GM-CSF may be responsible for the accumulation and differentiation of DCs in the bronchiolar tissues of patients with DPB. These results suggest that increased DCs in the bronchiolar tissues, together with their phenotypical maturation, may play an important role in the mucosal immune response in patients with DPB through their potent antigen-presenting function.

Dendritic cells (DCs) are the most potent antigen-presenting cells (APC) and play a central role in initiating primary immune responses (1, 2). DCs, widely distributed in virtually all organs except the brain, are preferentially in the mucosal surface, where they act as sentinels, sampling antigens and inducing immune responses against them (3). In the lung, DCs have been demonstrated to form a tightly meshed network in the epithelium of large airways (4). Thus, it is speculated that these airway DCs are strategically positioned to take up inhaled antigens, migrate to the local lymph nodes, and present the antigens to naive T cells in regional lymph nodes, leading to the initiation of an immune response in the lung. In addition, DCs are known to be very effective in stimulating memory T cells during secondary immune reactions. In the lungs of rodents and humans, alveolar macrophages were shown to have poor antigen-presenting function compared with DCs (5, 6). More recently, the suppressive regulatory role of alveolar macrophages has been emphasized (7). Thus, DCs are thought to be the most important APC in the lung because of their location and potent antigen-presenting capacity.

Recently, several studies have reported increased numbers of DCs in the large airways of patients with atopic asthma, suggesting that DCs play an important role in the inflammation of airways in patients with asthma (8–10). These findings suggest the possibility that the number of airway DCs might be increased under other pathological conditions causing chronic airway inflammation and that increased DCs may be involved in the mucosal immunity of these conditions via their potent antigen-presenting function. However, little is known about the number, distribution, and differentiated state of lung DCs in other lung diseases that occur in humans. Moreover, the distribution of DCs in the small airways remains to be determined, especially under pathological conditions.

Diffuse panbronchiolitis (DPB) is first described by Homma and coworkers as a chronic inflammatory disorder of the airways characterized histologically by peribronchiolitis with infiltration of lymphocytes and plasma cells (11–13). Patients with DPB usually present with productive cough, shortness of breath, and coarse crackles. Their chest X-rays show bilateral diffuse granular opacities predominantly in the lower lung fields. Pulmonary function tests reveal hypoxia and obstructive impairment. Because the bronchioles and their adjacent areas are primarily inflamed in patients with DPB, it is possible that the number of DCs in the small airways may be altered in patients with DPB. So far no report has described the distribution of DCs in the bronchiolar tissues of patients with DPB.

The purpose of this study is to elucidate the distribution of DCs in the bronchiolar tissues of patients with DPB and compare it with the distribution observed in control subjects with normal lungs, employing immunohistochemical studies with DC-specific antibodies.

METHODS

Patients

The study population included 11 patients with DPB who underwent an open lung biopsy or video-assisted thoracoscopic lung biopsy. The diagnosis of DPB was based on the clinical criteria established by Homma and Yamanaka and was confirmed histologically by the presence of the distinct pathological features including chronic inflammation with infiltration of mononuclear cells and an accumulation of foam cells around the bronchioles (11–13). There were seven men and four women, with a mean age of 50 yr. All of the patients with DPB were nonsmokers. As a control, lung tissues were obtained at the open thoracotomy from seven patients (four men and three women; with a mean age 58 yr) with primary lung cancer (n = 5), hamartoma (n = 1), and tuberculoma (n = 1). Lung tissues at a distance from the lesions, showing no abnormality, were used as normal lung tissues. All patients except one were nonsmokers.

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Histology and Tissue Processing

Lung biopsy specimens were obtained from at least one site in two or three lobes in each patient with DPB. In the group of control subjects, we took specimens at two different sites distant from the lesion. For light microscopic examination, lung tissues were fixed in 10% formaldehyde and embedded in paraffin. Then 4-μm-thick sections were cut and stained with hematoxylin and eosin. For immunohistochemical study, lung tissues were frozen by immersion in liquid N_2, embedded in O.C.T. Tissue-Tek (Miles, Elkhart, IN), and stored at −80° C. Frozen sections (4 μm) were cut on a cryostat (Bright, Huntington, UK) and placed on poly-L-lysine-coated microscopic slides. Sections were air dried for 1 h and stored at −80° C.

Immunohistochemistry

Immunohistochemical analysis was performed using a modified streptavidin–biotin–peroxidase complex method with a Histofine SAB-PO kit (Nichirei, Tokyo, Japan). The following monoclonal antibodies (mAb) were used: anti-CD1a (O10; Immunotech, Marseille, France), anti-CD1c (L161; Immunotech), anti-CD83 (HB15a, Immunotech), anti-granulocyte–macrophage colony-stimulating factor (GM-CSF) (126.2.1.3.2; Genzyme, Cambridge MA), and anti-CD68 (KP1, Zymed, CA). Briefly, 4-μm sections were fixed in acetone for 10 min at room temperature (RT). Nonspecific protein staining was blocked with goat serum. The slides were then treated with 0.3% hydrogen peroxidase to eliminate endogenous peroxidase for 20 min at RT, and incubated with the primary antibody for 1 h at RT, followed by biotinylated goat anti-mouse immunoglobulin antibody for 20 min at RT. The slides were then incubated with streptavidin–biotin–peroxidase complex for 15 min at RT. They were developed with 3-amino-9-ethylcarbazole (AEC) and counterstained with hematoxylin.

Quantitative Analysis of Positive Cells in Lung Tissues

To quantify the numerical densities of cells positive for staining with mAbs in the epithelium and submucosal tissue of bronchioles, images of sections were analyzed using a computer. Briefly, images of each section were made in a microscope (Vanox AHBS3; Olympus Corp., Tokyo, Japan) with a CCD camera (Fujix HC-2000; Fujifilm Corp., Tokyo, Japan). In each case, immunostained sections from two or three lung tissues were examined. Then, in each image, we counted the numbers of cells that were reactive with mAbs in the epithelium and submucosal tissue of bronchioles separately and also measured the length of the epithelial basement membrane corresponding to the evaluated area using an image analysis software (Mac Scope Version 2.1.7, Mitani Corp., Tokyo, Japan). Submucosal tissue of bronchioles was determined to be within an area of 200 μm lying beneath the epithelial basement membrane, excluding mucosal glands and vessels. In all samples, a region with a length of at least 5 mm was evaluated. The results were expressed as the mean numbers of positive cells per millimeter of epithelial basement membrane.

Statistics

For statistical analysis, the Mann–Whitney U test was used. A p value < 0.05 was considered significant. All data are expressed as mean ± SEM unless otherwise specified.

RESULTS

Clinical Characteristics of Patients with DPB

The clinical characteristics of the patients with DPB are summarized in Table 1. Chronic sinusitis was observed in all patients. All patients presented with dyspnea and productive cough with the duration of symptoms ranged from 8 to 295 mo, with a mean duration of 27.2 mo. Coarse crackles were audible in all patients. Nine (90%) of 10 patients tested revealed increased titers of cold agglutination (CHA). An elevation of serum immunoglobulin A (IgA) level was seen in five (32%) patients with a mean of 389 mg/dl. Blood gas analysis showed a decreased Pa_{O_2} in eight patients (73%) with a mean of 60 mm Hg. The levels of percent vital capacity (% VC) and FEV_1/FVC were reduced in 8 patients (73%) and 7 patients (64%), respectively.

Histologic Findings of Patients with DPB

In all patients with DPB, lymphocytes and plasma cells were found to have infiltrated the wall of the respiratory and membranous bronchioles, accompanied by accumulations of foam cells in the bronchial walls and surrounding alveolar septa (Figure 1A and B). These findings were consistent with those found in patients with DPB reported by Homma and coworkers (11–13). Seven of the patients with DPB had hyperplastic lymphoid follicles, which had characteristics of bronchus-associated lymphoid tissue (BALT) as described by Sato and coworkers (data not shown) (14).

Dendritic Cells in the Bronchiolar Epithelium

In the control subjects with normal lungs, $CD1a^+$ and $CD1c^+$ cells were present in the bronchiolar epithelium (1.20 ± 0.29

TABLE 1

CLINICAL CHARACTERISTICS

Diagnosis	Age (*yr*)	Sex (*M/F*)	Smoking	Chronic Sinusitis	Duration of Illness (*mo*)	CHA (X)	IgA (*mg/ml*)	Pa_{O_2} (*mm Hg*)	Pa_{CO_2} (*mm Hg*)	% VC	FEV_1 (%)
DPB	44	M	None	+	137	128	201	64.8	38.7	77.0	66.0
	57	M	None	+	120	512	1,200	71.6	40.0	51.5	77.9
	27	M	None	+	8	8	318	67.8	37.5	69.2	77.2
	45	M	None	+	124	128	302	70.7	40.7	82.1	65.2
	22	M	None	+	26	1,024	485	89.2	40.6	73.5	58.4
	52	M	None	+	52	256	402	57.4	40.4	63.5	61.9
	39	M	None	+	176	32	253	88.6	44.6	88.8	81.7
	52	F	None	+	15	1,024	435	64.7	29.7	67.1	62.3
	59	F	None	+	144	512	465	66.3	41.1	91.8	60.6
	62	F	None	+	44	512	213	68.0	35.4	79.0	76.0
	55	F	None	+	295	ND	255	87.5	34.7	68.0	66.0
Normal	69	M	Current	−	—	ND	ND	75.0	39.0	94.5	82.0
	73	M	None	−	—	ND	2,713	79.1	46.3	86.4	73.2
	41	M	None	−	—	ND	ND	97.1	39.0	107.0	80.8
	51	M	None	Unknown	—	ND	221	91.8	37.5	80.9	81.4
	61	F	None	−	—	ND	ND	90.0	42.0	81.2	79.5
	44	F	None	Unknown	—	ND	ND	96.8	40.3	121.8	71.8
	49	F	None	Unknown	—	ND	296	95.1	35.8	93.1	84.8

Definition of abbreviations: CHA = cold agglutination; ND = not done; IgA = immunoglobulin A; VC = vital capacity.

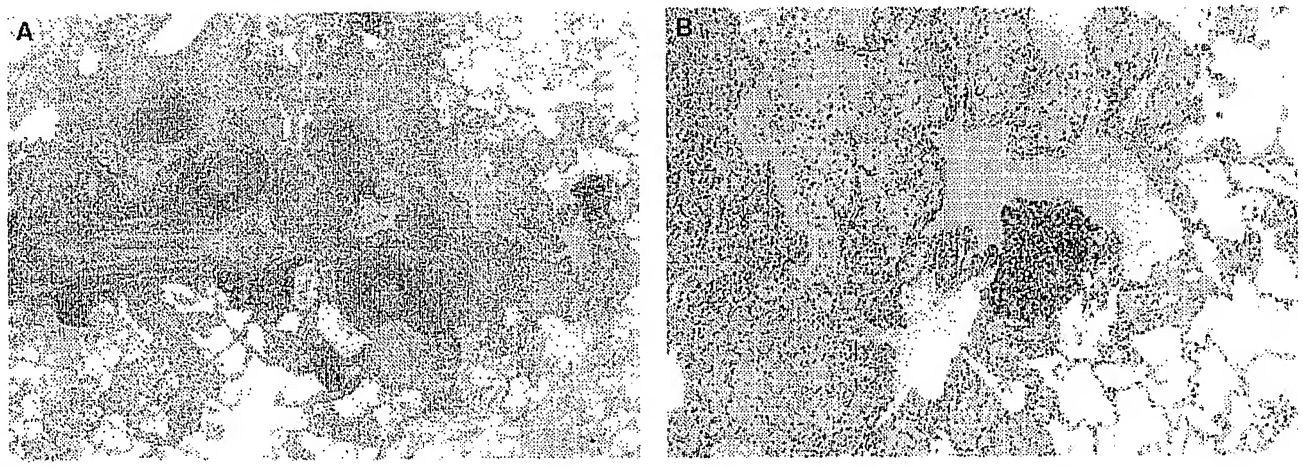

Figure 1. Cellular bronchiolitis with infiltration with small mononuclear cells (*A*, original magnification: ×16). Hyperplastic lymphoid follicles were observed around the bronchioles. Foam cells accumulated in the bronchiolar wall and surrounding alveolar septa (*B*, original magnification: ×40) (hematoxylin–eosin stain).

and 1.54 ± 0.54 cells/mm, respectively) (Figure 2). These cells, displaying a dendritic shape, were predominantly located just above the bronchiolar basement membrane (data not shown). There were few $CD83^+$ cells in the epithelium (0.04 ± 0.04 cells/mm) (Figure 2).

In the lungs of the patients with DPB, the number of $CD1a^+$ and $CD1c^+$ cells tended to increase in the bronchiolar epithelium (1.96 ± 1.20 and 4.61 ± 2.15 cells/mm, respectively) compared with control subjects (Figures 2 and 4). In addition, the number of $CD83^+$ cells was significantly higher in patients with DPB (0.50 ± 0.13 cells/mm) than in control subjects (Figures 2 and 4).

Dendritic Cells in the Submucosal Tissue of Bronchioles

$CD1a^+$ and $CD1c^+$ cells were also found in the bronchiolar submucosal tissues of the control subjects with normal lungs (0.19 ± 0.12 and 0.67 ± 0.40 cells/mm, respectively) (Figure 3). The number of $CD1a^+$ and $CD1c^+$ cells was almost equal in the epithelium, whereas in the submucosal tissues, $CD1c^+$ cells were more abundant than $CD1a^+$ cells (Figure 3). A very small number of $CD83^+$ cells was observed in the submucosal tissues of the control subjects with normal lungs (0.04 ± 0.04 cells/mm) (Figure 3).

In the patients with DPB, marked increases in the number of $CD1a^+$, $CD1c^+$, and $CD83^+$ cells with dendritic morphology were observed in the submucosal tissues compared with the control subjects (Figures 3 and 4). The number of $CD1a^+$ and $CD1c^+$ cells was significantly higher (17.0- and 13.9-fold, respectively) in patients with DPB (3.23 ± 0.58 and 9.36 ± 2.84 cells/mm, respectively) than in the control subjects (Figure 3). Interestingly, in the patients with DPB, we found a striking increase (117.5-fold) of $CD83^+$ cells (4.70 ± 1.73 cells/mm), which were considered to be pheotypically and functionally mature, in the bronchiolar submucosal tissues (Figures 3 and 4). Although the number of these three mAb-positive cells was higher in both the bronchiolar epithelium and submucosal tissues of the patients with DPB than in the control subjects, the degree of increase in the cell number was much larger in the submucosal tissues than in the epithelium.

Expression of GM-CSF Protein in the Bronchiolar Tissues

Normal bronchiolar epithelial cells were weakly but uniformly positive for anti-GM-CSF antibody (Figure 5). In patients with DPB, the bronchiolar epithelial cells strongly expressed GM-CSF protein (Figure 5). The intensity of its staining was much stronger in patients with DPB than in the control subjects.

Macrophages in the Bronchiolar Tissues

To compare the distribution of DCs with that of other antigen-presenting cells, we also evaluated the number of macrophages

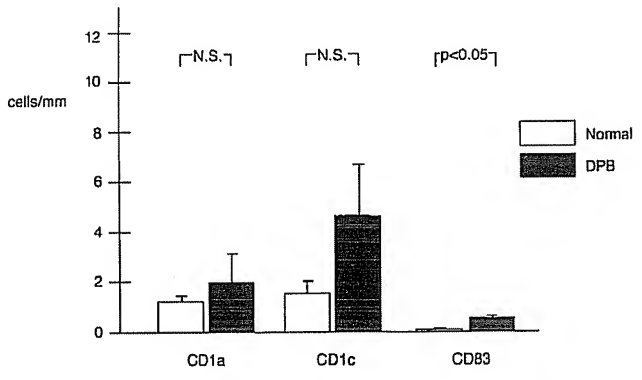

Figure 2. Number of $CD1a^+$, $CD1c^+$, and $CD83^+$ cells in the bronchiolar epithelium.

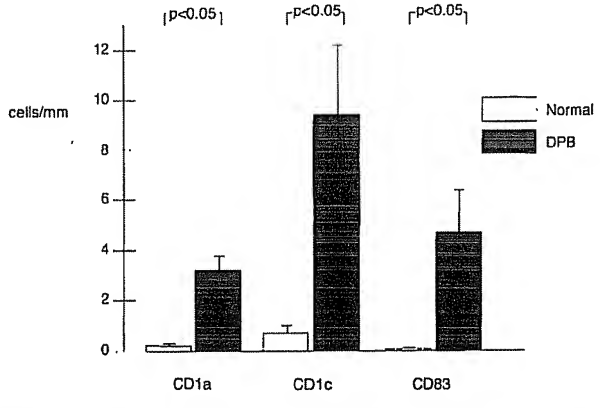

Figure 3. Number of $CD1a^+$, $CD1c^+$, and $CD83^+$ cells in the submucosal tissue.

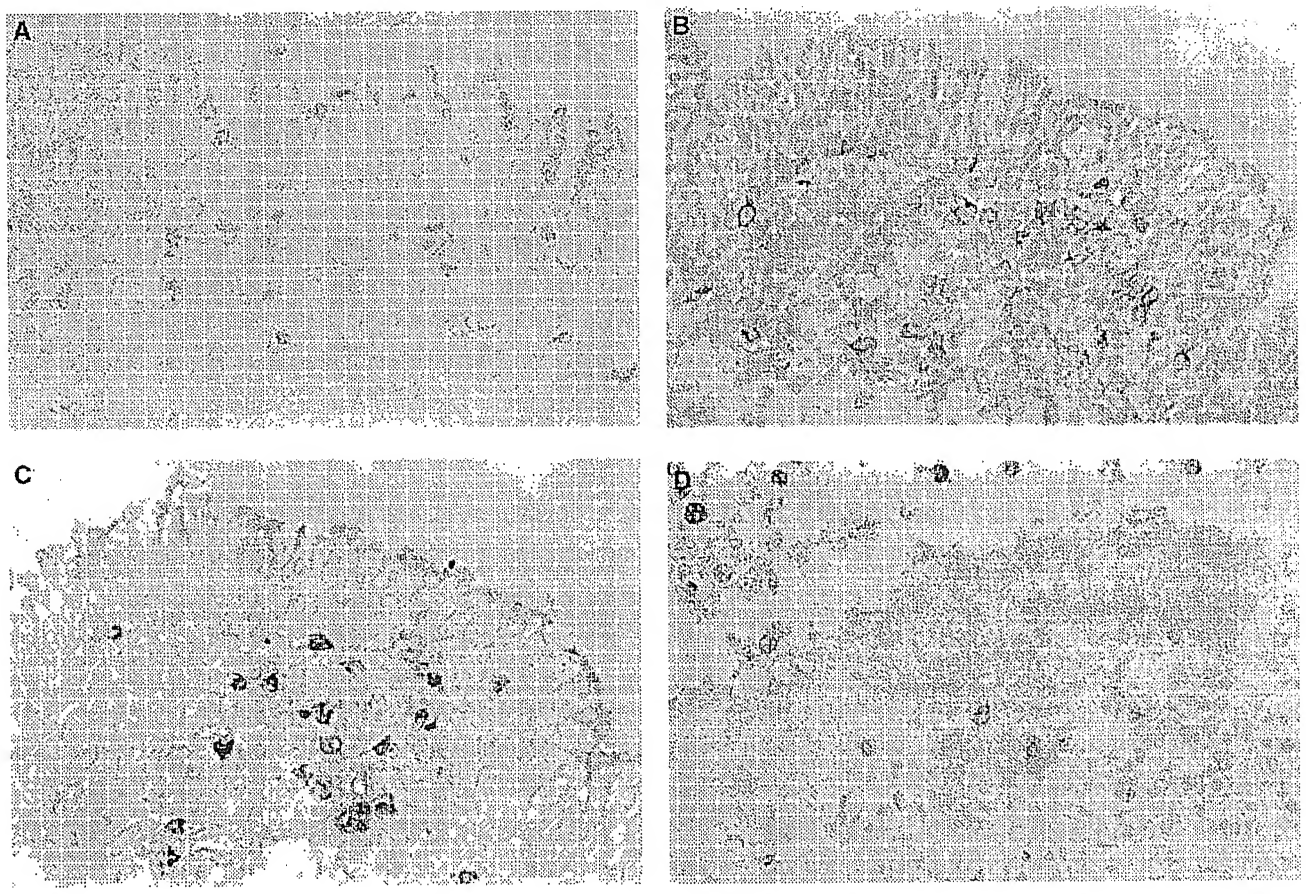

Figure 4. Immunostained bronchiolar tissue of patients with DPB. $CD1a^+$, $CD1c^+$, $CD83^+$, and $CD68^+$ cells are shown in bronchiolar tissue (*A*, *B*, *C*, and *D*, respectively, original magnification: ×100). $CD1a^+$, $CD1c^+$, and $CD83^+$ cells displaying a dendritic shape increased in submucosal tissue compared with bronchiolar epithelium.

in the bronchiolar tissues using anti-CD68 mAb. CD68, which is commonly used as a macrophage marker, is also expressed on small population of DC lineage cells (15, 16). However, its staining patterns differ between macrophages and DCs. Macrophages show homogeneous pancytoplasmic positivity for anti-CD68 mAb, whereas perinuclear dot-like staining is observed in DCs (17–19). Thus, cells uniformly stained in their cytoplasm with anti-CD68 mAb were counted as a macrophage. In the normal human lung, $CD68^+$ cells were absent in the bronchiolar epithelium and there were very few $CD68^+$ cells in the submucosal tissues of the bronchioles (0.01 ± 0.01 cells/mm). On the other hand, in the lungs of the patients with

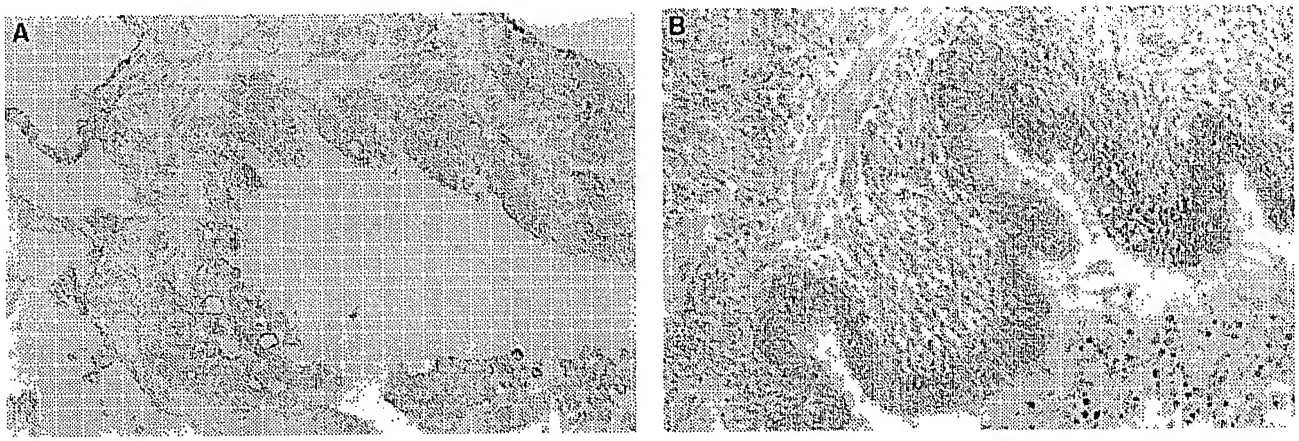

Figure 5. Expression of GM-CSF protein in the bronchiolar tissues. Normal bronchiolar epithelial cells were weakly positive for GM-CSF (*A*, original magnification: ×100), whereas those of patients with DPB strongly expressed GM-CSF protein (*B*, original magnification: ×100).

DPB, the number of $CD68^+$ cells markedly increased in both the epithelium and the submucosal tissues of the bronchioles (0.05 ± 0.04 cells/mm and 0.37 ± 0.23 cells/mm, respectively) (Figure 4), though their differences were not significant. In comparing the numerical density of macrophages with that of DCs in patients with DPB, the number of macrophages was markedly lower than the numbers of $CD1a^+$, $CD1c^+$, and $CD83^+$ DCs in the bronchiolar tissues of patients with DPB (macrophages versus $CD1a^+$ DCs, $p = 0.0503$, $p = 0.0076$; macrophages versus $CD1c^+$ DCs, $p = 0.0055$, $p = 0.0076$; macrophages versus $CD83^+$ DCs, $p = 0.0102$, $p = 0.0132$; in the epithelium and submucosal tissues, respectively).

DISCUSSION

In this study, we demonstrated that the number of DCs in the bronchiolar epithelium and submucosal tissues of patients with DPB was significantly higher than in control subjects with normal lungs. Additionally, the increases in the number of DCs were found to be more marked in the submucosal tissues than in the epithelium of the bronchioles.

In control subjects with normal lungs, we found that DC lineage cells expressing CD1a and CD1c were present in the bronchiolar tissues. Previous studies reported that $CD1a^+$ cells were mainly distributed within the bronchiolar epithelium, whereas $CD1c^+$ cells were predominant in the peribronchiolar tissue (20, 21). In this study, however, there was no significant difference in the number of $CD1a^+$ and $CD1c^+$ cells within the epithelium. In the submucosal tissues of the bronchioles, $CD1c^+$ cells predominated over $CD1a^+$ cells, consistent with previous reports. To date there is no report having examined the presence of $CD83^+$ cells in bronchiolar tissues. CD83 antigen is a newly established DC-specific marker that is expressed by mature DCs and can be used to delineate the maturation of cultured human blood DCs (22–24). We found few $CD83^+$ cells in the normal bronchiolar tissues.

In the bronchiolar tissues of patients with DPB having chronic inflammation predominantly around the bronchioles, marked increases in the number of $CD1a^+$, $CD1c^+$, and $CD83^+$ cells were observed in both the bronchiolar epithelium and submucosal tissues. Moreover, the increases of these positive cells in patients with DPB were more prominent in the submucosal tissues than in the epithelium (CD1a, 1.6-fold increase versus 17.0-fold increase; CD1c, 3.0-fold versus 13.9-fold; CD83, 12.5-fold versus 117.5-fold, in the epithelium and submucosal tissues, respectively, compared with the control subjects with normal lungs). Since an intense infiltration of T cells was present in the bronchiolar submucosal tissues of patients with DPB (13), there is a possibility that increased DCs in the submucosal tissues may present inhaled antigens and/or pathogens directly to the infiltrating T cells there, in addition to migrating to regional lymph nodes and stimulating T cells in the lymph nodes. More interestingly, we found the most striking increase in the number of $CD83^+$cells in the submucosal tissues of patients with DPB, whereas there were very few $CD83^+$ cells in the normal bronchiolar tissues. As previously described, $CD83^+$ cells are demonstrated to be very mature DCs, which express high levels of MHC class II antigens and adhesion molecules, such as CD80 and CD86, and have a strong antigen-presenting function (23, 24). Thus, marked accumulation of $CD83^+$ mature DCs in the submucosal tissues in patients with DPB could result in effective stimulation of the submucosal infiltrating T cells by their powerful antigen-presenting capacity.

In patients with asthma, the number of DCs in the large airways has also been reported to increase significantly over control subjects (8–10). In these studies, consistent with our results, the number of subepithelial DCs was significantly higher than that of intraepithelial DCs. Although the primarily affected sites of the airways differ between patients with DPB and patients with asthma, DCs are thought to accumulate predominantly in submucosal tissues in which T cells are infiltrating in both diseases.

Little is known about the factors governing the distribution and differentiated state of DC lineage in the lung. It has been reported that $S\text{-}100^+$ and/or $CD1a^+$ DCs accumulate within cancers in the lung (25, 26). Recently, several studies demonstrated a close correlation between the production of GM-CSF from cancers in the lung and the number of $CD1a^+$ DCs infiltrating the tumors (21). Moreover, in subjects with normal lungs, DCs were reported to be preferentially distributed at the sites at which GM-CSF was locally produced, such as the bronchiolar epithelium (21). Since GM-CSF has been demonstrated to have important effects on the differentiation and function of DCs (27–29), it is suggested that the local production of GM-CSF by the tumor cells or epithelial cells plays a crucial role in the recruitment and differentiation of DCs. In this study, we found that the bronchiolar epithelial cells in patients with DPB were strongly positive for GM-CSF, suggesting that local production of GM-CSF was enhanced in the bronchiolar tissues of patients with DPB. Since a variety of inflammatory stimuli are known to augment GM-CSF production by epithelial cells and other types of cells (30, 31), increased GM-CSF production by these cells in the inflamed bronchiolar tissues of patients with DPB appeared to be responsible for the accumulation and differentiation of DCs. Although normal bronchial epithelial cells produced GM-CSF, the amount of GM-CSF protein was much lower in control subjects with normal lungs than in patients with DPB, as assessed by its staining intensity. Recently several chemokine receptors were reported to be expressed by DCs, depending on their states of differentiation (32). Moreover, a variety of chemokines have been described as candidates of chemotactic factors for DCs (33, 34). Thus, it is likely that other chemokines released in the inflamed lung may also be involved in the accumulation of DCs in the bronchiolar tissues of patients with DPB. More works will be needed to determine which chemokine(s) play a central role in the increase of DCs in the bronchiolar tissues of patients with DPB.

To investigate the distribution of other antigen-presenting cells in the bronchiolar tissues of patients with DPB, we also examined the number of macrophages and compared it to that of DCs. Although there was a negligible number of macrophages in control subjects with normal bronchiolar tissues, the patients with DPB showed marked increases of $CD68^+$ macrophages in the epithelium and submucosal tissues of the bronchioles. However, the number of macrophages was markedly lower than the numbers of $CD1a^+$, $CD1c^+$, and $CD83^+$ DCs in the bronchiolar tissues of patients with DPB, suggesting that DCs were likely to be prominent antigen-presenting cells in the small airways of patients with DPB. It has been established that alveolar macrophages suppress T-cell proliferation as well as DC function (5–7). On the other hand, interstitial lung macrophages were shown to effectively activate T cells (35), indicating that the function of lung macrophages varies in their anatomical locations of the lung in terms of T-cell stimulatory capacity. However, little is known about the function of macrophages in the bronchiolar tissues. Further study is required to elucidate their precise roles in the mucosal immune response.

In summary, this study shows that the numbers of DC-lineage cells markedly increased in the bronchiolar tissues of patients with DPB together with their phenotypic maturation,

suggesting that these accumulated DCs may play an important role in the mucosal immune response against inhaled pathogens through their potent antigen-presenting function in patients with DPB.

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